Dual physiological effects of antifungal sterol biosynthetic inhibitors on enzyme targets and on transcriptional regulation

James H Crowley† and Leo W Parks*

Department of Microbiology, North Carolina State University, Box 7615, Raleigh, NC 27695-7615, USA

Abstract: For many antifungal agents, enzymes leading to ergosterol biosynthesis are primary targets. Inhibition of ergosterol biosynthesis in treated cells results in the formation of aberrant sterols, lacking one or more structural features of esgosterol. Furthermore, the total sterol levels are often higher than the total sterol amounts in non-treated cells. The *ERG3* gene, encoding the sterol C-5 desaturase, was used as a model for the regulation by some antifungal agents of genes encoding enzymes in ergosterol biosynthesis. Treatment of yeast cells with three sterol biosynthetic inhibitors with different targets in the ergosterol biosynthetic pathway led to an increase in *ERG3* mRNA levels. The increase in *ERG3* mRNA by drug treatment correlated with a decrease in ergosterol content within the cells. No correlation was evident between *ERG3* mRNA and total sterol levels, as treatment with at least one inhibitor, fenpropimorph, led to a slight increase in total sterol, while *ERG3* mRNA levels decreased. Treatment of cells with fenpropimorph and ketoconazole resulted in a decrease in ergosterol as a percentage of total sterol, while lovastatin caused an increase in the ergosterol percentage. These results indicate that a second indirect effect of the antifungal sterol biosynthetic inhibitors is on transcriptional regulation. The physiology of the treated cell is affected not only by a decrease in ergosterol but also by an enhanced accumulation of defective sterols.

Keywords: sterol biosynthesis inhibitors; yeast; ketoconazole; fenpropimorph; lovastatin; sterol regulation

1 INTRODUCTION

Ergosterol is the principal sterol of fungi. Sterol biosynthetic inhibitors (SBIs) inhibit one or more enzymatic reactions in ergosterol synthesis (Fig 1), resulting in the accumulation of non-ergosterol sterols and a concomitant decrease in intracellular ergosterol levels.[1,2] There are multiple functions for ergosterol,[3] and growth inhibition may be a combination of a depletion of ergosterol and the aberrant sterols which do not support one or more essential functions provided by ergosterol.

Recent studies have shown that ergosterol is able to regulate gene expression at the level of transcription.[4–6] *ERG3* is the structural gene for the sterol-C-5 desaturase which mediates the last ring modification in ergosterol biosynthesis.[7] We observed that strains of *Saccharomyces cerevisiae*, containing mutations in several of the late reactions, had elevated levels of *ERG3* mRNA and increased β-galactosidase activity when the mutant strains contained an *ERG3-lacZ* reporter fusion.[5] Strains with mutations in these *ERG* genes cannot synthesize ergosterol but do produce other sterols which will support growth at, however, a substantial physiological disadvantage.[8] The effects of sterol alterations on the physiology of yeast cells have been reviewed.[7] We were interested in determining more closely how sterol levels, as affected by sterol biosynthetic inhibitors, may control the expression of the *ERG3* gene, which is used as a model for regulation in ergosterol biosynthesis. In the present study, several different inhibitors of sterol biosynthesis were used to demonstrate that it is the levels of ergosterol that affect the regulation of *ERG3* gene expression, as opposed to the total level of sterols in yeast cells. Following treatment with the morpholine and azole sterol biosynthetic inhibitors, and probably others as well, there would be an ergosterol deficiency. In addition, the cell would be flooded with the aberrant sterols.

† Present address: Monsanto, 800 N Lindbergh Blvd, U4D St. Louis, MO 63167, USA
* Correspondence to: Leo W Parks, Department of Microbiology, North Carolina State University, Raleigh NC 27695-7615, USA
E-mail: parks@mbio.ncsu.edu
Contract/grant sponsor: National Institutes of Health.
Contract/grant No: DKL 37222
Contract/grant sponsor: North Carolina Agricultural Research Service

(*Received 15 June 1998; revised version received 30 September 1998; accepted 2 December 1998*)

 Pestic Sci 0031-613X/99/$17.50

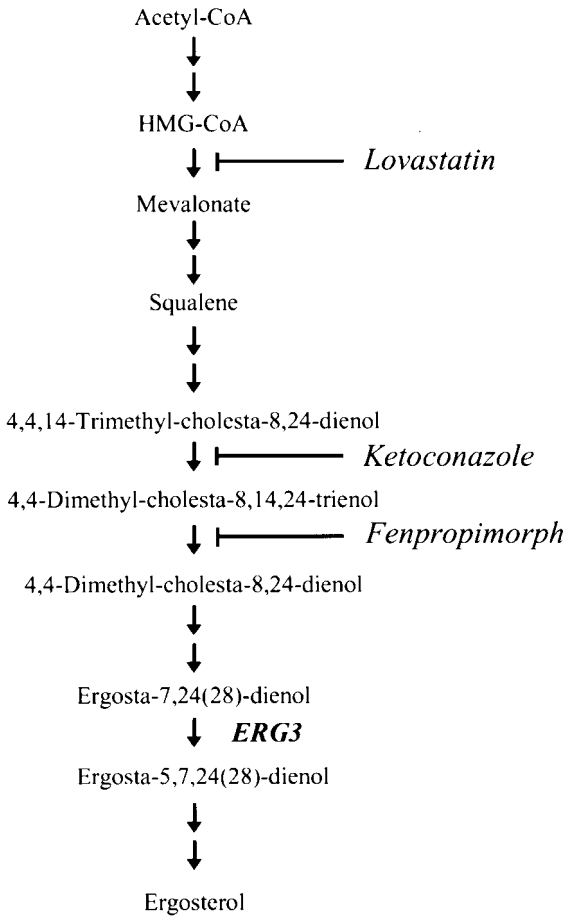

Figure 1. Sites of inhibition of the pathway of ergosterol synthesis in *Saccharomyces cerevisiae* by the sterol biosynthesis inhibitors lovastatin, ketoconazole, and fenpropimorph. The location of the *ERG3* gene, encoding the C-5 sterol desaturase is also indicated.

2 MATERIALS AND METHODS

2.1 Yeast strains and growth conditions

The yeast strain used in this study was *S cerevisiae* strain SY114 (a *ura3*-52, *his3 trp1*).[5] Yeast cultures were grown on a synthetic complete medium with 50 mM succinate buffered to pH 5.5 with potassium hydroxide. For addition of sterol biosynthesis inhibitors, cultures were grown overnight in synthetic complete media and inoculated to fresh medium to $OD_{600} = 0.5$, and fenpropimorph, ketoconazole, or lovastatin was added to the indicated concentrations. Stock solutions of fenpropimorph and ketoconazole were prepared at 10 mg ml^{-1} in ethanol, and a stock solution of lovastatin (20 mg ml^{-1}) was prepared according to the method of Lorenz and Parks.[9] Solvent concentration did not exceed 7.5 ml litre^{-1} and ethanol was added to control cultures of SY114 to this concentration.

2.2 mRNA analysis

Cultures were grown to mid-log phase (OD_{600} = 1.0) and total RNA was harvested as described previously.[5] Ten micrograms of total RNA were loaded from each sample into 1.5% formaldehyde-agarose gels and separated by electrophoresis for Northern blot analysis.[10] Northern blots were hybridized to radiolabeled DNA probes of the *ERG3* and *ACT1* coding regions.

Hybridizations were carried out as described previously[5] with hybridizations to *ERG3* first and then hybridizations to *ACT1* following the stripping of the *ERG3* probe according to the manufacturer of the GeneScreen nylon membranes (Dupont-NEN Research Products, Boston, MA). Northern blots were analyzed both by autoradiography and imaging by a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Quantitation of the band intensity was performed with the PhosphorImager and corrections for RNA loading errors were made based on the *ACT1* control hybridization.

2.3 Preparation and analysis of sterols

Cultures were grown overnight in 50 ml of synthetic complete medium and inoculated into 375 ml of fresh medium to an optical density at 600 nm of 0.5 (approximately 1 : 10 dilution), and inhibitors were added at this time. The cultures were allowed to grow at 30°C until mid-log phase (optical density at 600 nm of 1.0) and were harvested by centrifugation, washed in distilled water, and lyophilised. The dried cell pellets were weighed and total sterols were liberated by saponification and extracted with hexane.[11] Cholesteryl ester was added prior to saponification as an internal standard for identification of sterols and for monitoring extraction efficiency. Sterols were separated by gas chromatography as described by Fenner and Parks[12] and quantitation of sterols was performed based on a standard curve of the peak areas of cholesterol.

2.4 Materials

Dextrose, YNB (yeast nitrogen base without amino acids), and extraction solvents were from Fisher Scientific. Amino acids, nucleotide bases, and ketoconazole were from Sigma Chemical Co. GeneScreen nylon membranes for Northern blots and the radiolabeled nucleotide, [α-^{32}P]dCTP, were from Dupont-NEN. Random-primed DNA labeling kits from Boehringer Mannheim were used to make radiolabeled DNA probes for Northern analyses. Fenpropimorph was a gift from BASF (Ludwigshafen, Germany) and lovastatin was a gift from A Alberts (Merck Research Laboratories, Rahway, NJ).

3 RESULTS AND DISCUSSION

The effects of sterol biosynthetic inhibition on *ERG3* gene expression in *S cerevisiae* were examined. Lovastatin, ketoconazole, and fenpropimorph were used as inhibitors. The sites of action of these three inhibitors are depicted in Fig 1. Lovastatin (mevinolin) is an inhibitor of HMG-CoA reductase in mammalian and yeast cells,[9,13] and treatment of cells with lovastatin starves the cell not only for

sterol, but also for other isoprenoid compounds such as dolichols, ubiquinones, heme A, farnesylated proteins and isoprenyl tRNA. As such, growth inhibition of cells with lovastatin is not fully reversed by supplementation with sterol. Ketoconazole is a member of the azole family of antifungal agents and is an inhibitor of the product of the *ERG11* gene in yeast, the cytochrome *P*450-requiring enzyme, C-14 sterol demethylase.[14] Treatment with ketoconazole results principally in the accumulation of lanosterol and 14α-methyl-cholestra-8,24-dien-3,6-diol, which are not suitable sterols for the growth of *S cerevisiae*.[15] Fenpropimorph is a member of the morpholine class of antifungal agents, and fenpropimorph inhibits both the C-8,7 sterol isomerase and C-14 sterol reductase, resulting in the accumulation of ergosta-8,14-dienol (ignosterol).[16,17] This mode of action is similar to that of 15-azasterol, which is also a potent antifungal agent that results in accumulation of ignosterol.[18,19] This is an unsuitable sterol for growth of *S cerevisiae* under some conditions.[20]

To compare directly the *ERG3* mRNA levels and sterol levels of drug-treated cells, cultures were grown in increasing concentrations of inhibitors, and an aliquot of the culture was used for extraction of RNA; the remaining portion was used for sterol analysis. Treatment of *S cerevisiae* strain SY114 with increasing concentrations of lovastatin, ketoconazole, and fenpropimorph led to an increase in the levels of *ERG3* mRNA to as much as 19-fold in comparison to the untreated control culture (Fig 2). The increase in steady-state levels of *ERG3* mRNA due to sterol alterations is known to occur as a function of increased transcription of the *ERG3* gene.[5] A dose-dependent increase in *ERG3* expression was evident for both fenpropimorph- and ketoconazole-treated cultures; however, an equivalent high level of derepression of the *ERG3* gene by lovastatin was observed under the range of lovastatin concentrations used.

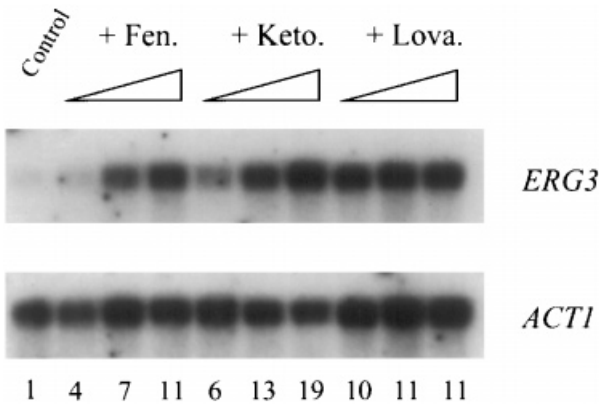

Figure 2. Northern analysis of *Saccharomyces cerevisiae* strain SY114 treated with fenpropimorph (Fen.), ketoconazole (Keto.), or lovastatin (Lova.). Cultures were grown overnight and inoculated into fresh synthetic complete medium with increasing concentrations of inhibitors or without inhibitor (control). The concentrations used were (in $\mu g\,ml^{-1}$) 0.01, 0.1, 1.0 fenpropimorph; 0.05, 0.5, 5.0 ketoconazole; and 5.0, 50 and 150 lovastatin. Northern analysis on total RNA was performed as described in Section 2.2 with probes made to the coding regions of *ERG3* and *ACT1* (Actin 1 gene; control for RNA loading errors). The fold increase in *ERG3* mRNA levels is reported below the Northern analysis in relation to the untreated control. Autoradiograms of the Northern blots were scanned by using a Relysis Image Scanner and Adobe Photoshop version 2.5 for Macintosh.

The sterol contents of the treated cultures were analyzed to determine if there is a relationship between sterol levels and *ERG3* expression. Sterols were extracted and saponified so that the total amounts of free and esterified sterols could be quantified in order to understand how the total sterol pools may affect gene expression. The reduction in ergosterol as a consequence of SBI treatment is consistent with the increase in *ERG3* expression (Fig 3). A reduction in cellular ergosterol content was observed for both fenpropimorph and ketoconazole treatments and less reduction in ergosterol content was achieved with lovastatin. Unlike fenpropimorph and ketoconazole, lovastatin also blocks the synthesis of other terpenoids such as heme A, and heme does affect regulation of ergosterol synthesis.[7] Even though a dose-dependent response in ergosterol reduction was not observed for lovastatin, a reduction of ergosterol was consistent with a concomitant increase in *ERG3* expression. Although the data do not allow consistent predictions of *ERG3* expression levels based on ergosterol content, we believe that the reduction of ergosterol levels, mediated by the inhibitors, is a principal determinant in *ERG3* expression.

Even though wild-type yeast produces a mixture of sterol molecules during active growth, ergosterol is selectively positioned in the membrane and other non-ergosterol sterols are esterified[21,22] and partitioned to the lipid bodies along with triacylglycerol molecules.[23] It remained plausible that the increase

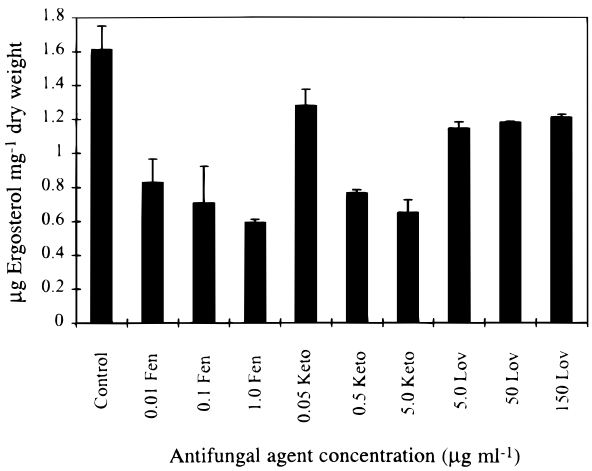

Figure 3. Effects of sterol biosynthetic inhibitors on ergosterol levels in *Saccharomyces cerevisiae*. Cultures were grown overnight and inoculated into fresh synthetic complete medium with or without the addition of fenpropimorph (Fen.), ketoconazole (Keto.), or lovastatin (Lov.). Sterols were extracted from yeast cultures and quantified as described in Section 2.3. Error bars represent the standard deviation from three individual samples.

in *ERG3* expression, seen in the inhibitor-treated cultures, might be due to a reduced total sterol content rather than to a specific decrease in ergosterol. An analysis of the total sterol in the cultures revealed inconsistencies between total sterol levels and *ERG3* expression (Fig 4). Treatment with fenpropimorph did not alter the total amount of sterol within the cell and even produced a slight increase in sterol content. Ketoconazole inhibition did result in a decrease in sterol content, but the level of sterol decrease was similar with different concentrations, which is unlike the dose-dependent decrease in ergosterol with ketoconazole treatment. The total sterol levels as a consequence of lovastatin exposure did correspond with the increase in *ERG3* expression, however, under these circumstances, ergosterol made up 100% of the sterol species found (Fig 5).

In comparing the percentage of ergosterol in the total sterol in the treated cultures, the different modes of action of the three SBIs are evident (Fig 5). In treatment with fenpropimorph, accumulation of ignosterol is at the expense of ergosterol, and the total amount of sterol increases. Ketoconazole treatment reveals a more complicated scenario where 14-methylated sterols accumulate at the expense of ergosterol with an additional loss of total sterol until a maximum is reached. This indicates that ketoconazole may inhibit another site within the sterol biosynthetic pathway, prior to the C-14 sterol demethylase. In consequence, all sterol synthesis was slightly reduced. The increase in ergosterol as a percentage of total sterols under the lowest treatment with ketoconazole was consistent with this idea. A restriction early in the pathway before squalene synthesis reduces all sterol levels such that only ergosterol is made, and other precursor sterols, which are routinely observed in untreated yeast cultures, are absent. This is seen in the case of lovastatin treatment, where inhibition of HMG-CoA reductase reduces total sterol, and ergosterol comprises 100% of the sterol in comparison to the usual 52% found in untreated wild-type cells.

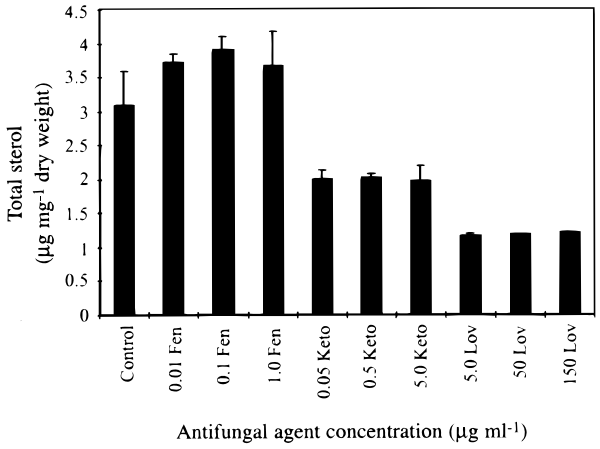

Figure 4. Effects of fenpropimorph, ketoconazole, and lovastatin on total sterol levels in *Saccharomyces cerevisiae*. Cultures were grown overnight and inoculated into fresh synthetic complete medium with or without the addition of fenpropimorph (Fen.), ketoconazole (Keto.), or lovastatin (Lov.). Sterols were extracted from yeast cultures and quantified as described in Section 2.3. Error bars represent the standard deviation from three individual samples.

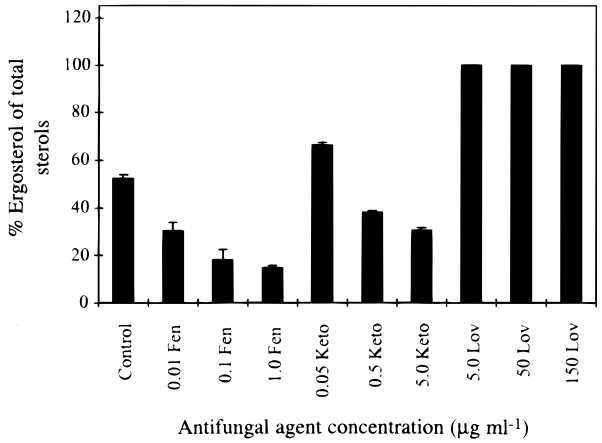

Figure 5. The percentage of ergosterol in total sterols produced by *Saccharomyces cerevisiae* treated with sterol biosynthetic inhibitors. Cultures were grown overnight and inoculated into fresh synthetic complete medium with or without the addition of fenpropimorph (Fen.), ketoconazole (Keto.), or lovastatin (Lov.). Sterols were extracted from yeast cultures and quantified as described in Section 2.3. Error bars represent the standard deviation from three individual samples.

Of particular interest is understanding the mechanism of how ergosterol levels can ultimately regulate gene expression. Our results do not establish that ergosterol is the sole effector in regulating sterol synthesis, however. In mammalian cells, cholesterol is believed to regulate gene expression at least in part not through cholesterol itself but through an oxysterol derived from cholesterol.[24] Although oxysterols have not been found in yeasts, it remains to be seen if a physiologically significant sterol derivative may function in controlling gene expression in yeast. Our attempts to control *ERG3* expression through sterol feeding have thus far been unsuccessful (unpublished observations). A complication in those feeding experiments is that wild-type yeast cells did not take up sterol aerobically. Mutants that can take sterols from the medium have grossly altered sterol physiology.[7] Nevertheless, studies on the regulation of ergosterol biosynthesis may reveal vulnerable targets for both antifungal intercession and sterol-lowering drugs.

ACKNOWLEDGEMENTS

This research was supported in part by a grant from the National Institutes of Health (DKL37222) and the North Carolina Agricultural Research Service. The authors thank BASF (Ludwigshafen, Germany) for a gift of fenpropimorph and Merck Research Laboratories (Rahway, NJ) for a gift of lovastatin.

REFERENCES

1 Burden RS, Cooke DT and Carter GA, Inhibitors of sterol biosynthesis and growth in plants and fungi. *Phytochemistry*, **28**:1791–1804 (1989).

2 Mercer EI, Inhibitors of sterol biosynthesis and their applications. *Prog Lipid Res* **32**:357–416 (1993).
3 Rodriguez RJ, Low C, Bottema CDK and Parks LW, Multiple functions for sterols in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **837**:336–343 (1985).
4 Arthington-Skaggs BA, Crowell DN, Yang H, Sturley SL and Bard M, Positive and negative regulation of a sterol biosynthetic gene (*ERG3*) in the post-squalene portion of the yeast ergosterol pathway. *FEBS Lett* **392**:161–165 (1996).
5 Smith SJ, Crowley JH and Parks LW, Transcriptional regulation by ergosterol in the yeast, *Saccharomyces cerevisiae*. *Mol Cell Biol* **16**:5427–5432 (1996).
6 Dimster-Denk D and Rine J, Transcriptional regulation of a sterol-biosynthetic enzyme by sterol levels in *Saccharomyces cerevisiae*. *Mol Cell Biol* **16**:3981–3989 (1996).
7 Parks LW and Casey WM, Physiological implications of sterol biosynthesis in yeast. *Annu Rev Microbiol* **49**:95–116 (1995).
8 Palermo LM, Leak FW, Tove S and Parks LW, Assessment of the essentiality of *ERG* genes late in ergosterol biosynthesis in *Saccharomyces cerevisiae*. *Curr Genet* **32**:93–99 (1997).
9 Lorenz RT and Parks LW, Effects of lovastatin (mevinolin) on sterol levels and on activity of azoles in *Saccharomyces cerevisiae*. *Antimicrob Agent Chemother* **34**:1660–1665 (1990).
10 Brown T and Mackey K, Analysis of RNA by northern and slot blot hybridization. *Curr Protoc Mol Biol* **1**:4.9.1–4.9.16 (1997).
11 Parks LW, Bottema CDK, Rodriguez RJ and Lewis TA, Yeast sterols: yeast mutants as tools for the study of sterol metabolism. *Methods Enzymol* **111**:333–346 (1985).
12 Fenner GP and Parks LW, Gas chromatographic analysis of intact steryl esters in wild-type *Saccharomyces cerevisiae* and in an ester-accumulating mutant. *Lipids* **24**:625–629 (1989).
13 Alberts AW, Chen J, Kuron G, Hunt C, Hoffman C, Rothrock J, Lopez M, Joshua H, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hirchfield J, Hoogsteen K, Liesch J and Springer J, Mevinolin: a highly potent competitive inhibition of hydroxymethylglutaryl-CoA reductase and a cholesterol-lowering agent. *Proc Nat Acad Sci USA* **77**:3957–3961 (1980).
14 Van den Bossche H, Lauwers W, Willemsens G, Marichal P, Cornelissen F and Cools W, Molecular basis for the antimycotic and antibacterial activity of *N*-substituted imidazoles and triazoles: the inhibition of isoprenoid biosynthesis. *Pestic Sci* **15**:188–198 (1984).
15 Watson PF, Rose ME, Ellis SW, England H and Kelly SL, Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole anifungals. *Biochem Biophys Res Commun* **164**:1170–1175 (1987).
16 Baloch RI, Mercer EI, Wiggins TE and Baldwin BC, Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. *Phytochemistry* **23**:2219–2226 (1984).
17 Baloch RJ and Mercer EI, Inhibition of sterol 8-7 isomerase and 14-reductase by fenpropimorph, tridemorph, and fenpropidin in cell-free systems from *Saccharomyces cerevisiae*. *Phytochemistry* **26**:663–668 (1987).
18 Hays PR, Parks LW, Pierce HD Jr and Oelschlager AC, Accumulation of ergosta-8,14-dienol by *Saccharomyces cerevisiae* cultured with an azasterol antimycotic agent. *Lipids* **12**:666–668 (1997).
19 Bottema CDK and Parks LW, 14-sterol reductase in *Saccharomyces cerevisiae*. *Bichim Biophys Acta* **531**:301–307 (1978).
20 Crowley JH, Smith SJ, Leak FW and Parks LW, Aerobic isolation of an *ERG24* null mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **178**:2991–2993 (1996).
21 Bailey RB and Parks LW, Yeast sterol esters an their relationship to the growth of yeast. *J Bacteriol* **124**:606–612 (1975).
22 Taylor FR and Parks LW, An assessment of the specificity of sterol uptake and esterification in *Saccharomyces cerevisiae*. *J Biol Chem* **256**:13048–13054 (1981).
23 Zinser E, Paltauf F and Daum G, Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J Bacteriol* **175**:2853–2858 (1993).
24 Kandutsch AA, Chen HW and Heiniger HJ, Biological activity of some oxygenated sterols. *Science* (*Washington*) **201**:498–501 (1978).